**ABSTRACT**

**Objectives:** The current classification of inflammatory bowel disease (IBD) is based on clinical phenotypes, which is blind to the molecular basis of the disease. The aim of this study was to stratify a treatment naïve paediatric IBD cohort through specific innate immunity pathway profiling and application of unsupervised machine learning (UML).

**Methods:** In order to test the molecular integrity of biological pathways implicated in IBD, innate immune responses were assessed at diagnosis in 22 paediatric patients and 10 age-matched controls. Peripheral blood mononuclear cells (PBMCs) were selectively stimulated for assessing the functionality of upstream activation receptors including NOD2, toll-like receptor (TLR) 1-2 and TLR4, and the downstream cytokine responses (IL-10, IL-1β, IL-6 & TNF-α) using multiplex assays. Cytokine data generated were subjected to hierarchical clustering to assess for patient stratification.

**Results:** Combined immune responses in patients across twelve effector responses were significantly reduced compared to controls (p=0.003) and driven primarily by ‘hypofunctional’ TLR responses (p values 0.045, 0.010 & 0.018 for TLR4-mediated IL-10, IL-1β & TNF-α respectively; 0.018 & 0.015 for TLR1-2 -mediated IL-10 & IL-1β). Hierarchical clustering generated three distinct clusters of patients and a fourth group of ‘unclustered’ individuals. No relationship was observed between the observed immune clusters and the clinical disease phenotype.

**Conclusions:** Although a clinically useful outcome was not observed through hierarchical clustering, our study provides a rationale for using an UML approach to stratify patients. The study also highlights the predominance of hypo-inflammatory innate immune responses as a key mechanism in the pathogenesis of IBD.

**Key Words:** Inflammatory bowel disease, innate immunity, hierarchical clustering

**What is known?**

* Dysregulated immune responses are the key drivers of inflammation in inflammatory bowel disease (IBD).
* The current clinical classification of IBD is blind to the underlying immunological phenotype or the molecular basis of the disease.
* Treatment strategies in IBD are largely guided by the clinical phenotype, disease progression and course.

**What is new?**

* Children with IBD can be stratified into sub-groups or clusters based on their immune response profiles using an unsupervised machine learning approach.
* A predominance of hypo-inflammatory toll-like receptor (TLR) responses is seen in a significant number of paediatric IBD patients at diagnosis.

**INTRODUCTION**

Inflammatory bowel disease (IBD) is a cytokine-mediated auto-inflammatory condition, comprising three main clinical phenotypes, Crohn’s disease (CD), ulcerative colitis (UC) and IBD-unclassified (IBDU)1. The aetiology of this polygenic immune-mediated disease is complex with a combination of multiple factors converging to produce similar clinical phenotypes. Current treatment protocols are driven by clinical phenotypes which are blind to the molecular basis of the disease, resulting in sub-optimal outcomes and escalation of treatment strategies, sometimes necessitating invasive surgical procedures, with consequential life-long morbidity2 3. Identifying sub-groups of patients with specific immune defects will facilitate targeted therapeutics based on the molecular characterisation and stratification of the disease.

The primary aim of this study was to profile specific innate immune responses in peripheral blood mononuclear cells (PBMCs) in a treatment naïve paediatric IBD cohort and assess if patients could be stratified based on their induced cytokine responses. The reason for using treatment naïve samples was to minimise the masking impact of drug interventions on immune responses.

The cytokine data generated were subjected to hierarchical clustering to assess patient-stratification based on immune response profiles. Hierarchical clustering is a well-researched application of unsupervised machine learning, which seeks to identify natural groupings within a complex data set by reducing variable dimensionality and clustering data points with similar patterns1.

The study focused on the functional integrity of innate signalling pathways of known biological importance in IBD mediated via the NOD2, toll-like receptor (TLR)-1-2 and TLR44 5, measuring effector cytokine responses using multi-plex assays. **Figure 1** provides a schematic representation of the molecular basis of the assay.

NOD2 receptor is an intra-cellular pathogen recognition receptor (PRR), which senses specific conserved fragments such as muramyl dipeptide (MDP), a peptidoglycan motif found in the cell wall of several gram-positive and gram-negative bacteria6. Upon recognition of MDP, NOD2 receptor sub-units undergo self-oligomerisation, recruiting receptor-interacting serine-threonine-protein kinase 2 (RIPK2) through their caspase recruitment domain (CARD). Further downstream, RIPK2 mediates recruitment of other adaptor proteins and kinases, which are essential for the activation of the nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPK) and the Nod-like receptor family pyrin domain 3 (NLRP3)-inflammasome cascades7. Unlike NOD2, which is an intra-cellular receptor, TLRs such as TLR1, TLR2 and TLR4 are cell-surface receptors. TLRs recognize pathogen associated molecular patterns (PAMPs) such as lipids, lipoproteins and other microbial products. Mutations in several TLR genes have been implicated in the pathogenesis of IBD. The most well-studied among the TLRs for their association with IBD include TLR2 and TLR48 9. Following interaction with their corresponding stimulants, TLRs interact with adaptors such as myeloid differentiation primary response protein 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF), which then activate downstream signalling cascades. Activation of signalling cascades such as NF-κB, MAPK and NLRP3- mediated inflammasome activation pathways results in the transcription of cytokines including IL-10, IL-6, IL-1β and TNF-α, which can be quantified through multiplex assays10 11. Cytokine analysis following activation of specific receptors indicates the functionality of proteins involved in these pathways, from the stimulated receptor to the specific cytokine produced10 11.

**METHODS**

**Study participants**

Children under 18 years of age, with suspected IBD were recruited prior to an established diagnosis over a 12-month period from January 2016 to January 2017. Twenty-two patients diagnosed with IBD following an upper and lower GI endoscopy were included in the study. The diagnosis was made in line with the ESPGHAN revised Porto criteria for IBD12. Specimens from ten children with normal endoscopic and histological assessment of the gastro-intestinal biopsies were used as non-IBD controls. The non-IBD control group underwent investigations in view of non-specific GI symptoms, but had normal investigations, remained well at six months follow up and were subsequently discharged from the service. Blood samples for PBMC extraction from all participants were obtained prior to commencement of any treatment.

**PBMC extractions, activation and Luminex® assays**

PBMCs were extracted by density gradient centrifugation (Ficoll-PaqueTM Plus, GE Healthcare, Uppsala, Sweden) using up to 10 mls of whole blood per individual, collected in lithium heparin vacutainer tubes. Isolated PBMCs were cryopreserved in liquid nitrogen. For activation, PBMCs were used at a concentration of 0.3 x 105 cells, stimulated with Pam3CysSerLys4 (Pam3CSK4; TLR1-2 receptor stimulant; Invivogen, UK; dose- 10μg/ml), lipopolysaccharide (LPS; TLR4 stimulant; Sigma-Aldrich, UK; dose- 1μg/ml) and muramyl-dipeptide (MDP; NOD2 stimulant; Invivogen, UK; dose- 10μg/ml) for 24 hours. The cellular supernatants were analysed to measure the concentration of IL-10, IL-1β, IL-6 and TNF-α using Luminex® assays as per the manufacturer’s protocols (Invitrogen, User Manual: Human Cytokine 10-Plex Panel. Catalog no. LHC0001, California, USA). Luminex® assays are flow cytometry based multiplexing assays, measuring several analytes of interest using spectrally encoded magnetic beads, each with a specific fluorescent signature 13. All samples were assayed in duplicate. Monocyte counts were assessed using whole blood in every individual and the cytokine measurements standardised to a monocyte count of 10%. As there were three activating stimulants and four cytokines analysed, a total of twelve cytokine responses were assessed per individual.

***NOD2* variants**

Patients were assessed for *NOD2* variants using exome sequencing data. Any variation within the *NOD2* gene was extracted from the variant call files generated for each of the pIBD patients and categorised in line with the American College of Medical Genetics (ACGM) guidance to remove ‘benign’ variants and identify ‘pathogenic’ and ‘likely pathogenic’ variants14. Further details on the sequencing analysis are presented in the online supplementary material.

**Statistical analysis and cytokine data normalisation**

Statistical analysis was performed using the GraphPad Prism software, version 7 and SPSS (version 25, IBM). Cytokine induction between the patient cohort and controls were compared using unpaired t-tests (two-tailed). For the application of machine learning algorithms, raw cytokine data expressed in a conventional format (picograms/ml) were normalised using RobustScaler and StandardScaler within the python Scikit-Learn package15. Further details on data transformation and scaling statistics of these software tools are presented in the online supplementary material.

**Hierarchical clustering**

Hierarchical clustering was performed on the normalised data using the R software package16. An agglomerative hierarchical clustering (bottom-up approach) algorithm was used, which applies pairwise distance matrix between the observations as clustering criteria. The distances were calculated using Euclidean metrics and average linkage. A tree-based representation of agglomerative clustering starts at the bottom by treating each individual as a singleton cluster and at each step of the algorithm, the two individuals with the most similar profiles are merged, with pairs of clusters successively merged into one big cluster (root) to include the entire cohort of patients. In this study, clusters within the dendogram were identified using a ‘static’ tree-cut method, which defines each contiguous branch below a fixed height cut-off as a separate cluster16.

**Ethics statement**

Ethical approval was granted by Southampton & South West Hampshire Research Ethics Committee (09/H0504/125). Informed consent was obtained from the parents/legal guardians of all participants before recruitment to the study. Informed consent was also obtained from older children who were deemed capable of understanding the nature of the study.

**RESULTS**

**Patients and controls**

Twenty-two paediatric patients with IBD were included; 55% males (n=12), median age at diagnosis of 13 years (range 5-16 years); Crohn’s disease (n=14) and ulcerative colitis (n=8). The mean (+/- SD) paediatric Crohn’s disease activity index (PCDAI) and paediatric ulcerative colitis activity index (PUCAI) scores at the time of sample procurement were 37 (+/- 12.23) and 54.38 (+/- 12.94) respectively, reflecting a moderate-severe disease activity at diagnosis. The control group included 10 individuals, 60% males with a median age of 14 years (range 4-16 years). For patient demographics, see **table S1** in the online supplemental digital content (SDC).

**Induced immune responses on a radar plot**

Normalised cytokine values per patient per stimulus were plotted along the equi-angular spokes or radii of a radar plot, each representing a specific cytokine response (**figure 2**). Cytokine data from paediatric controls were used to define the reference ranges in the patient cohort. Values within 2 standard deviations (SD) from the mean cytokine values in controls were considered within normal response limits and, those above and below 2 SD indicated hyper-inflammatory and hypo-inflammatory responses respectively. None of the patients showed responses in the hyper-inflammatory range (>2 SD). The majority of cytokine values fell between the mean and -2 SD, suggesting a trend towards hypo-inflammatory responses.

**Hierarchical clustering**

In order to identify patterns or trends within the cytokine data across the twenty-two patients, hierarchical clustering of normalised cytokine profiles was used. With a static height cut-off of 1.25, the dendogram generated three distinct clusters of patients based on the computed similarity of their induced immune profiles and an ‘unclustered’ group of ten patients (grouped as cluster 4). See **figure 3.** The clustering pattern generated using this height cut-off was also distinctly recognisable through visual inspection of the dendogram. See **figure S1 in the supplementary**. All patients within each cluster had similar immune profiles, however no relationship was observed between the clusters and the clinical disease phenotype or disease severity. Multivariable linear regression did not demonstrate a significant association between any of the twelve cytokine responses and normalised disease severity scores at diagnosis. See online supplementary data (**table S2**).

**Visualisation of cluster patterns using radar plots**

In order to visualise the cytokine response patterns within individual clusters, radar plots of cytokine responses were generated per patient. Visual inspection of radar plots as groups within each cluster showed similar cytokine profiles, consistent with the stratification observed through an unsupervised machine learning approach, thereby supporting the underlying immunological basis of the clustering pattern. For example, patients in cluster 1 had comparatively reduced induction along the vectors for LPS-induced IL-1β & TNF-α, MDP-induced IL-6 & TNF-α and Pam3CSK4-induced IL-1β & TNF-α. The online supplemental digital content includes individual radar plots on patients in clusters 1-3 (**figure S2. A-C**).

**Comparing pooled cytokine data between the patient cohort and controls**

Normalised cytokine values for all 22 patients and 10 controls are presented in **table 1**. Raw cytokine data are presented in the online SDC **table S3**. The final column in table 1 represents pooled values obtained after summation of all the 12 cytokine responses per individual. There was a significant difference in the pooled responses between patients and controls (p=0.003), indicating a patient cohort signal of overall reduced immune responses. The combined reduced responses were driven predominantly by the toll-like receptor (TLR)-mediated responses [TLR4-induced IL-10 (p=0.045), IL-1β (p=0.010) & TNF-α (p=0.018) and TLR1-2-induced IL-10 (p=0.018) & IL-1β (p=0.015)]. There was no significant contribution from the NOD2-mediated responses.

**MDP-induced immune responses in the context of *NOD2* Variants**

Five *NOD2* variants were identified in eight individuals within the cohort, of which seven individuals had CD and one UC. Two of the variants included R702W and G908R, widely known to be associated with CD17. The other variants included disease-associated mutations R708H, R713H and V955I, categorised in line with the ACGM guidance14. One individual with CD (SPR339) had compound heterozygosity for G908R and R708H, as confirmed through segregation analysis. MDP-induced immune responses in this individual fell between the mean and -2 SD. The remaining seven individuals were heterozygous for the *NOD2* variants. Combined normalised cytokine values for MDP-induced immune responses showed no significant differences between the individuals with *NOD2* variants and those without. Furthermore, no significant differences were observed between the individuals with *NOD2* variants and the controls. See online supplementary data (**table S4**).

**Assessing inter-cluster differences**

In order to determine if the five significantly reduced cytokine responses observed across the twenty-two patients compared to controls were specific to individual patient clusters rather than the whole group, the within cluster values were compared against the control group using unpaired t-tests. This was conducted in order to qualitatively and statistically analyse how each cluster differed from the control group in terms of their dysfunctional cytokine responses. Cluster 1 showed significant differences for four out of five, cluster 2 for three out of five cytokine responses compared to controls and cluster 3 showed only borderline significant differences for TLR1-2 mediated IL-10 induction (p=0.047). See **table S5** in the online supplementary. T-tests were not conducted for the ‘unclustered’ group of patients (cluster 4).

**Cytokine profiles in CD and UC**

Normalised cytokine data between CD and UC patients were compared using unpaired t-tests. No significant differences were observed between the two phenotypes. See **table S6** in the online supplementary.

**DISCUSSION**

This study adopted a unique approach of first assessing the immunological phenotype of a treatment naïve paediatric cohort through selected innate immunity pathway profiling, followed by patient stratification through unsupervised machine learning. The study showed a significantly reduced cytokine induction in the patient cohort compared to controls, which was driven predominantly by hypo-inflammatory TLR-mediated responses, with no significant contribution from NOD2-mediated responses. No significant differences were observed in MDP-induced immune responses between individuals harbouring potentially pathogenic *NOD2* variants and those without the *NOD2* variants. Application of unsupervised machine learning approaches such as hierarchical clustering to the cytokine data enabled stratification of patients into distinct clusters based on their induced immune profiles. Generation of radar plots per individual for visual representation of the cytokine data demonstrated similar patterns within clusters, thereby providing a supportive conceptual evidence of the immunological basis of the hierarchical clustering.

Multiple studies assessing induced immune function have been described previously, however, the vast majority have been conducted in adult IBD patients following an established diagnosis and treatment18. In our study, immunological assays were performed in treatment naïve paediatric IBD patients where PBMCs were obtained at or before diagnosis, thereby removing the potential impact of drug interventions on immune responses. Previously published studies have clearly demonstrated the effect of drug treatments on the functioning of immunological assays19 20.

The reduced cytokine induction observed in this study may reflect signalling defects in the key inflammatory pathways resulting in impaired cytokine production. Reduced or absent innate immune induction has been reported previously, particularly in the context of known IBD-associated mutations in genes such as *NOD2*21 22. The findings from our study, combined with the weight of evidence from previously published studies21 22 suggest that poor or maladaptive immune response to pathogenic insults, with a consequential poor bacterial clearance and persistence of secondary inflammation may be a key driving mechanism in the pathogenesis of IBD. Another possible mechanism to explain the reduced cytokine induction observed in our patient cohort is down regulation of cytokine production in PBMCs due to the inhibitory effect of stromal factors secreted by the inflamed bowel tissue including TGF-β and IL-1023-25.

In this study, hypo-inflammatory immune responses were driven predominantly by reduction of TLR-mediated responses. TLRs are cell-surface or endosomal pathogen-recognition receptors, widely expressed in the GI epithelium. Alterations in TLR expression have been associated with the pathogenesis of IBD26. TLR2 forms a hetero-dimer with TLR1 or TLR6, which is necessary for signal transduction. Current evidence indicates a protective role for TLR1-TLR2 mediated responses27. Results have shown that poor expression of TLR1 during acute infection can lead to chronic bowel inflammation and dysbiosis. Experimental evidence suggests that a TLR2 focussed agonistic approach could hold promise for future therapeutic applications in IBD. On the other hand, although TLR4 signalling is protective against invading bacteria, it may trigger or aggravate mucosal inflammation through release of nitric oxide with consequent pro-inflammatory cytokine induction. Although the precise mechanistic influence of TLR4 on IBD pathogenesis is unknown, current evidence is indicative of a bi-directional role27.

Although CD and UC are distinct clinical entities with possibly different immunological mechanisms, all patients in this study were grouped together with the primary objective of exploring novel groupings within the cohort based on immunological profiles regardless of the clinical diagnosis. The binary classification of IBD into CD and UC although clinically very useful, is blind to the immunological phenotype of the disease28. It may be argued that given the complex polygenic nature of IBD and the resultant biological heterogeneity, the two conditions represent a phenotypic spectrum of an underlying immune dysregulation29. Whilst there are defined diagnostic criteria for CD and UC, there is frequently a histological overlap and subsequent re-classification of the disease sub-type particularly in children30. There is also a significant overlap in the genetic susceptibility to IBD, with patients frequently having both disease sub-types in their family history31. Furthermore, diverse genetic mutations can present with similar clinical phenotypes adding further challenges to the understanding of the genetic nature of the disease32.

Our study was limited by modest cohort size and profiling of a limited set of innate immune cytokine responses. To confirm wider applicability of the study findings, implementation of larger cohorts and more extensive immune profiling is required. Furthermore, as all patients had active inflammation at diagnosis when the samples were collected, it is unclear if the observed hypo-functionality is an epiphenomenon of inflammation. To follow this prospectively, it is important to demonstrate a maintained ‘cluster signature’ by using PBMCs collected from patients during remission, whilst not on any treatment that would impact on the assay function. However, it would be practically challenging to identify a sizable cohort of patients with controlled disease, but not on immunosuppressive treatment. Although specific immune pathway defects were not identified in this study, the observed clustering pattern suggests that there is a potential for stratifying patients based on immunological profiling with a futuristic goal of implementing targeted treatments.

Despite the emergence of robust multi-omic technologies over the last few years, there is an unmet need for novel groupings in complex immune-mediated conditions like IBD. The concept of machine learning to classify IBD into novel categories based on the disease biology is exciting and a clear steer towards personalised medicine1 31. The scope and clinical relevance of this approach in IBD is ever expanding with the advancements in the field of immune therapies and treatment with biologics. As a futuristic model of precision medicine, patient stratification on the basis of immunological and multi-omic profiling will lead to the development of tailored treatment strategies with optimal outcomes and minimal side-effects.

**Acknowledgements:** The authors are grateful to all patients and their families. We thank Nikki J Graham for technical assistance in the DNA laboratory. We also thank Dr Kathy Potter, Jenna Watt and the technical team from tissue bank for providing consistent laboratory support.

**REFERENCES**

1. Mossotto E, Ashton JJ, Coelho T, et al. Classification of Paediatric Inflammatory Bowel

Disease using Machine Learning. Sci Rep 2017;7(1):2427.

2. Ruemmele FM, Veres G, Kolho KL, et al. Consensus guidelines of ECCO/ESPGHAN on

the medical management of pediatric Crohn's disease. J Crohns Colitis 2014;8(10):1179-207.

3. Turner D, Levine A, Escher JC, et al. Management of pediatric ulcerative colitis: joint

ECCO and ESPGHAN evidence-based consensus guidelines. J Pediatr Gastroenterol Nutr

2012;55(3):340-61.

4. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease.

Nature 2011;474(7351):307-17.

5. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic

architecture of inflammatory bowel disease. Nature 2012;491(7422):119-24.

6. Inohara N, Ogura Y, Fontalba A, et al. Host recognition of bacterial muramyl dipeptide

mediated through NOD2. Implications for Crohn's disease. J Biol Chem 2003;278(8):5509

12.

7. Caruso R, Warner N, Inohara N, et al. NOD1 and NOD2: signaling, host defense, and

inflammatory disease. Immunity 2014;41(6):898-908.

8. Wang F, Tahara T, Arisawa T, et al. Genetic polymorphisms of CD14 and Toll-like

receptor-2 (TLR2) in patients with ulcerative colitis. Journal of gastroenterology and

hepatology 2007;22(6):925-9.

9. Hume GE, Fowler EV, Doecke J, et al. Novel NOD2 haplotype strengthens the association

between TLR4 Asp299gly and Crohn's disease in an Australian population. Inflammatory

bowel diseases 2008;14(5):585-90.

10. Oeckinghaus A, Ghosh S. The NF-kappaB family of transcription factors and its

regulation. Cold Spring Harbor perspectives in biology 2009;1(4):a000034.

11. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-kappaB signaling pathways.

Nature immunology 2011;12(8):695-708.

12. Levine A, Koletzko S, Turner D, et al. ESPGHAN revised porto criteria for the diagnosis

of inflammatory bowel disease in children and adolescents. J Pediatr Gastroenterol Nutr

2014;58(6):795-806.

13. Prabhakar U, Eirikis E, Miller BE, et al. Multiplexed cytokine sandwich immunoassays:

clinical applications. Methods Mol Med 2005;114:223-32.

14. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of

sequence variants: a joint consensus recommendation of the American College of Medical

Genetics and Genomics and the Association for Molecular Pathology. Genet Med

2015;17(5):405-24.

15. Oliphant TE. SciPy: Open source scientific tools for Python. Comput Sci Eng 2007;9:10

16. Langfelder P, Zhang B, Horvath S. Defining clusters from a hierarchical cluster tree: the

Dynamic Tree Cut package for R. Bioinformatics 2008;24(5):719-20.

17. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat

variants with susceptibility to Crohn's disease. Nature 2001;411(6837):599-603.

18. Coelho T, Andreoletti G, Ashton JJ, et al. Immuno-genomic profiling of patients with

inflammatory bowel disease: a systematic review of genetic and functional in vivo studies of

implicated genes. Inflamm Bowel Dis 2014;20(10):1813-9.

19. Wong SH, Gao Q, Tsoi KK, et al. Effect of immunosuppressive therapy on interferon

gamma release assay for latent tuberculosis screening in patients with autoimmune diseases:

a systematic review and meta-analysis. Thorax 2016;71(1):64-72.

20. Edwards A, Gao Y, Allan RN, et al. Corticosteroids and infliximab impair the

performance of interferon-gamma release assays used for diagnosis of latent tuberculosis.

Thorax 2017;72(10):946-49.

21. van Heel DA, Ghosh S, Butler M, et al. Muramyl dipeptide and toll-like receptor

sensitivity in NOD2-associated Crohn's disease. Lancet 2005;365(9473):1794-6.

22. Netea MG, Ferwerda G, de Jong DJ, et al. The frameshift mutation in Nod2 results in

unresponsiveness not only to Nod2- but also Nod1-activating peptidoglycan agonists. J Biol

Chem 2005;280(43):35859-67.

23. Smythies LE, Sellers M, Clements RH, et al. Human intestinal macrophages display

profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin

Invest 2005;115(1):66-75.

24. Babyatsky MW, Rossiter G, Podolsky DK. Expression of transforming growth factors

alpha and beta in colonic mucosa in inflammatory bowel disease. Gastroenterology

1996;110(4):975-84.

25. de Waal Malefyt R, Abrams J, Bennett B, et al. Interleukin 10(IL-10) inhibits cytokine

synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J

Exp Med 1991;174(5):1209-20.

26. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of

toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun

2000;68(12):7010-7.

27. Lu Y, Li X, Liu S, et al. Toll-like Receptors and Inflammatory Bowel Disease. Front

Immunol 2018;9:72.

28. Torres J, Colombel JF. Genetics and phenotypes in inflammatory bowel disease. Lancet

2016;387(10014):98-100.

29. Olivera P, Danese S, Jay N, et al. Big data in IBD: a look into the future. Nat Rev

Gastroenterol Hepatol 2019.

30. Rialon KL, Crowley E, Seemann NM, et al. Long-term outcomes for children with very

early-onset colitis: Implications for surgical management. J Pediatr Surg 2018;53(5):964-67.

31. Ashton JJ, Mossotto E, Ennis S, et al. Personalising medicine in inflammatory bowel

disease-current and future perspectives. Transl Pediatr 2019;8(1):56-69.

32. Cleynen I, Boucher G, Jostins L, et al. Inherited determinants of Crohn's disease and

ulcerative colitis phenotypes: a genetic association study. Lancet 2016;387(10014):156-67.

**Figure Legends**

**Figure 1**

 **Conceptual framework of the immunological assay**

LPS binds to TLR4, Pam3CSK4 to TLR1/2 and MDP to NOD2. Binding of the ligands to the

respective receptors result in the recruitment of adaptor proteins, which in turn activate the

signalling pathways including NF-κB, MAPK and the NLRP3- inflammasome, leading to

transcription of effector cytokines. The adaptor proteins including IAPs (XIAP, cIAP1 and

cIAP2) play a key role in signal transduction by engaging with other proteins for processes

such as ubiquitination and providing a scaffold for recruiting other effector kinases

downstream. (***Abbreviations:*** *cIAP, cellular inhibitors of apoptosis; IRAK4, interleukin-1*

*receptor associated kinases 4; MAPK, mitogen activated protein kinases; Myd88, myeloid*

*differentiation primary response protein 88; NF-κB, nuclear factor-κB; RIP2, receptor*

*interacting response protein 2; TRAF6, TNF-receptor associated factor 6; XIAP, X-linked*

*inhibitor of apoptosis*)

**Figure 2**

**Radar plot showing cytokine responses of all patients**

The ligands used for stimulation are depicted outside the boundary of each sector. The cytokines analysed are indicated within the boundary of each sector. The red-dotted line represents the mean obtained from paediatric controls. Responses within 2 SD across the mean are within the range of normality. Every patient is represented by a unique colour. Individual control subjects are not represented in this figure. The numbers 0, 1.0, 2.0 and 3.0 along each vector indicate the mean, 1 SD, 2 SD and 3 SD respectively.

**Figure 3**

**Hierarchical clustering to identify immuno-phenotypes**

The figure shows a Cluster heat map based on induced immune profiles in 22 paediatric patients. The maximum and minimum normalised cytokine values are indicated in red and blue respectively on the heat map. The probands are indicated on the X-axis and the immune profiles on the right Y-axis. Distances were calculated using Euclidean metrics and average linkage. Cluster identification was performed using a static height cut-off method. Hierarchical clustering shows three distinct clusters of patients and an ‘unclustered’ group of 10 patients (cluster 4).

**Table 1. Tabular matrix showing normalised cytokine data**

Normalised cytokine data following stimulation with the receptor-specific ligands including LPS (TLR4 stimulant), MDP (NOD2 stimulant) and Pam3CSK4 (TLR1-2 stimulant). Induction of 4 cytokines including IL-10, IL-1β, IL-6 and TNF-α were assessed per stimulant, thereby generating 12 assay conditions in total. The column at the extreme right represents summation of the values across all the cells in each row. The bottom row includes p values obtained by applying a 2-tailed student t-test across the values between the patient cohort and controls for the 12 cytokine responses. The t-tests were significantly different for 5 TLR-mediated responses (highlighted in red).

**Supplemental digital content (SDC)- Figure legends**

**Figure S1. Static height cut-off for cluster identification**

The figure demonstrates three distinct clusters generated using a static height cut-off at 1.25. Clusters 1, 2 and 3 had five, four and three patients respectively. Ten individuals fell in the ‘unclustered’ group at the applied height cut-off.

**Figure S2 (A-C). Individual Radar plots**

This figure shows immune response profiles for the patients in clusters 1-3 on individual radar plots. Patients within a cluster are put together and indicated by a unique colour. Each radar plot has 3 sectors representing a stimulant with the 4 cytokines analysed per sector. The red-dotted line is the mean obtained from cytokine responses observed in the control group.

**Supplemental digital content (SDC)- Table legends**

**Table S1. Patient cohort characteristics**

Patient characteristics are presented along with the PCDAI (mild 10-29, moderate-severe >30) and PUCAI (mild 10-34, moderate 35-64, severe >65) scores for CD and UC respectively. Disease location/s as per Paris classification for Crohn’s disease include L1 (distal 1/3 of ileum), L2 (colonic), L3 (ileo-colonic) & L4 (upper GI disease; L4a- proximal to the ligament of Treitz, L4b- distal to the ligament of Treitz); UC E1(ulcerative proctitis), E2 (left-sided UC), E3 (extensive, up to hepatic flexure) & E4 (pancolitis). NA- not available.

**Table S2. Regression analysis between immune profiles and disease activity scores**

Multi-variable linear regression was performed between the twelve cytokine responses and the normalised disease activity scores. The disease activity scores were normalised as the scoring tools are different in CD and UC. There was no evidence of a correlation between the immune profiles and the disease activity scores.

**Table S3. Raw cytokine data**

The table shows raw cytokine data prior to normalisation. Effector cytokine responses were assessed following stimulation of the PBMCs with the receptor-specific ligands including LPS (TLR4 stimulant), MDP (NOD2 stimulant) and Pam3CSK4 (TLR1-2 stimulant). Induction of four cytokines including IL-10, IL-1β, IL-6 and TNF-α were assessed per stimulant, thereby generating 12 assay conditions in total.

**Table S4. *NOD2* variants and MDP-induced immune responses**

The table shows *NOD2* variants identified in eight patients alongside the MDP-induced immune responses. The p values for t-tests comparing MDP-mediated immune responses between individuals with *NOD2* variants and those without are indicated in the bottom row.

*[Abbreviations: ACMG- American College of Medical Genetics (individual variant classification for evidence of pathogenicity include: VS- very strong, S- strong, M- moderate, Su- supporting); het- heterozygote; hg19- human genome version 19; HGMD- The Human Gene Mutation Database (DFP- disease associated mutation with functional evidence; ? DM- disease causing mutation, the question mark indicates a degree of uncertainty of the pathogenic potential of the mutation based on updated reports on HGMD)]*

**Table S5. Comparing the five dysfunctional cytokine responses between each cluster and controls**

The 5 dysfunctional TLR-mediated responses were compared between each cluster and the control group using unpaired t-tests. The table shows p values obtained after comparing the respective clusters against the control samples. T-tests were not performed in the ‘unclustered’ group (cluster 4).

**Table S6. Comparing immune profiles in CD and UC**

Normalised cytokine data following stimulation with the receptor-specific ligands including LPS (TLR4 stimulant), MDP (NOD2 stimulant) and Pam3CSK4 (TLR1-2 stimulant). Induction of 4 cytokines including IL-10, IL-1β, IL-6 and TNF-α were assessed per stimulant, thereby generating 12 assay conditions in total. The column at the extreme right represents summation of the values across all the cells in each row. The bottom row includes p values obtained by applying a 2-tailed student t-test across the values between patients with CD and UC. No significant differences were observed.